Rapid Enzyme-Induced Hydrolysis of Microgram Amounts of Phosphatidylcholine on Phospholipase A_2/Celite Columns[1,2]

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ABSTRACT

A method has been developed to hydrolyze microgram amounts of phosphatidylcholine (PC) regiospecifically. Hydrolysis of the *sn*-glycerol 2-acyl group occurs rapidly on microcolumns of immobilized phospholipase A_2 on Celite 545 diatomaceous earth. Close to 90% reaction occurs within the first 5 min. Acyl group analysis then may be accomplished by gas liquid chromatography (GLC) of the resulting fatty acids. Hydrolysis of α-16:0-β-14:0 PC demonstrated consistent selectivity for 14:0 liberation, with small amounts of 16:0 probably indicative of acyl scrambling during the synthesis of the PC. Hydrolysis of an equal weight mixture of $(14:0)_2$PC and $(18:2)_2$PC demonstrated nonpartiality of the immobilized enzyme for either a saturated or unsaturated substrate. The new methodology offers a convenient and sensitive alternative to the presently used procedures.

INTRODUCTION

Phospholipase A_2 (phosphatide 2-acyl hydrolase, E.C. No. 3.1.1.4) specifically catalyzes the hydrolysis of the *sn*-glycerol 2-acyl group of glycerophosphatides such as phosphatidylcholine (PC) to yield a free fatty acid and a lysoglycerophosphatide (1,2).

Standard methodology for such hydrolyses requires multiphase contact between an ether phase containing milligram amounts of lipid and a buffered aqueous phase containing the enzyme and the necessary calcium ion cofactor. Generally the heterogeneous mixture is shaken at least 1 hr, followed by extraction of the lipid residue, separation of the liberated free fatty acid, LPC, and unreacted PC by thin layer chromatography (TLC), esterification of the fatty acid or transesterification of the LPC, and finally analysis of the resulting methyl esters by gas liquid chromatography (GLC) (3-6).

The present research was influenced by recent reports on the immobilization of lipase enzymes (7-10) and the conviction that analysis of *microgram* amounts of lipids is a feasible, rapid, and convenient alternative to conventional methodology. Prior work demonstrated that microgram amounts of phosphatidylcholine could be analyzed for total acyl group content by transmethylation on microcolumns of potassium methoxide/Celite diatomaceous earth (11). It was envisioned, therefore, that phosphatidylcholine could be analyzed for its 2-acyl group by hydrolysis on microcolumns containing immobilized phospholipase A_2. Immobilization by adsorption of an aqueous solution of the enzyme onto Celite would allow a large surface area of the aqueous enzyme to contact the lipid in organic phase.

EXPERIMENTAL

Preparation of Enzyme Solution

Phospholipase A_2 (toxic; handle with care) from *Crotalus terrificus terrificus* venom was obtained in 50% aqueous glycerol, 1 mg/ml, activity 200 μ/mg (Boehringer Mannheim GmbH). This was diluted 1:1 with a Tris buffer solution that was adjusted to pH 7.5 by addition of hydrochloric acid and made 4 mM in calcium ion. The buffer solution was prepared by dissolving Tris (J.T. Baker Chemical Co., Phillipsburg, NJ, $(HOCH_2)_3CNH_2$, 0.605 g) in water (25.0 ml), then adding 0.1 N hydrochloric acid (40.6 ml) and $CaCl_2 \cdot 2H_2O$ (39 mg). The resulting "working solution" of the enzyme was similar in constitution to that in the modified Brockerhoff procedure reported by Christie (6).

Celite Microcolumn

Large Pasteur pipettes were shortened to give glass capillaries with built-in funnels, as reported previously (11). A small plug of glass wool was inserted two-thirds the way down the capillary, and a 1.5 cm microcolumn of carefully washed (4 x by conc. HCl, 4 x by distilled water) Celite 545 diatomaceous earth (Fisher Scientific Co., Pittsburgh, PA) was tamped in place above the plug. Measured aliquots of the phospholipase A_2 working solution (generally 2 μl) were carefully added to the top of the Celite within the column by microliter syringe. If de-

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[1]Presented at the AOCS 68th Annual Meeting, New York, May 1977.

[2]Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

TABLE I

Regioselectivity of Enzymic Hydrolysis of α-16:0 -β-14:0 PC

Enzyme working solution, μl	Reaction time, min	Number of experiments	% 14:0[a]	mole % 14:0[b]
2	5	8	88.8 ± 1.6	90.1
2	30	5	88.2 ± 0.7	89.6
4	5	4	88.4 ± 1.0	89.8
4	30	4	86.8 ± 1.2	88.4
6	60	3	89.6	90.6

[a]Flame ionization detector response as mean ± standard deviation; [14:0/(14:0 + 16:0)] x 100.

[b]FID response is proportional to n-1 carbon atoms in a C_n fatty acid (15).

sired, these columns then could be sealed at both ends with a small flame and stored in a freezer for later use. They retained their activity for at least several months when prepared and stored this way.

Preparation of Lipid Solution

Synthetic L-α-phosphatidylcholines served as model compounds: α-palmitoyl-β-myristoyl- (Supelco, Inc., Bellefonte, PA); dimyristoyl- (Sigma Chemical Co., St. Louis, MO); dilinoleoyl- (PL Biochemicals, Inc., Milwaukee, WI). Solutions were prepared in Nanograde methylene chloride (2.5 μg/μl), purities were confirmed by TLC (11), and concentrations were determined by phosphorus analysis (12). Individual solutions of $(14:0)_2$PC and $(18:2)_2$PC of known concentrations were combined to give a new solution that contained equal weights of each lipid.

Reaction Conditions

A prepacked enzyme microcolumn was snapped open at both ends. The lipid solution (1.0 to 10 μl) was deposited onto the column with a 10 μl syringe. The reaction was allowed to proceed at room temperature (generally 5 min for GLC analysis of released free fatty acid, but up to 1 hr for maximum hydrolysis). Then the liberated free fatty acids were eluted from the column with a portion of ether (30-50 μl). This eluate was free of contaminants (by TLC) and ready for GLC analysis. In order to establish the extent of reaction, the residual material was eluted with methanol (50 μl) and analyzed by TLC.

Gas Liquid Chromatography

Product mixtures of myristic and palmitic acids were analyzed directly on an EGA/phosphoric acid column using conditions identical to those reported earlier for methyl esters (11). Mixtures of myristic and linoleic acids were converted to their methyl esters by treatment with an ether solution of diazomethane prior to GLC analysis (13,14).

Thin Layer Chromatography

Commercial plates of Silica Gel H and 5% ammonium sulfate were used (Redi-Coat L/S, Supelco, Inc., Bellefonte, PA). Plates were developed in one direction in order to separate the reactant PC from the product LPC. Optimum development required a predevelopment with acetone and then the use of chloroform-methanol-acetic acid-water, 25:15:4:2 by volume. Plates were visualized by charring in an oven at 180 C, at which temperature the incorporated charring agent, ammonium sulfate, pyrolyzed to sulfuric acid. Phosphorus-containing spots could also be visualized by spraying the plate with a diluted molybdate spray (12) or with Phospray (Supelco). Nondestructive visualization was accomplished by contact with iodine vapor.

Quantitative Analysis of PC and LPC

The Vaskovsky-Kostetsky-Vasendin phosphorus analysis procedure was used (12). The heteropolyphosphomolybdate blue was measured in a Bausch and Lomb Spectronic 88 spectrophotometer at 830 nm; values were compared to KH_2PO_4 standards. In typical runs, PC and LPC TLC spots were scraped and digested in 0.2 ml 70% $HC10_4$ in a block heater at 180 C for about 20 min. These data were compared with values obtained for blank scrapings of equivalent R_f and for identical aliquots of the reagent PC solution.

RESULTS AND DISCUSSION

Separation of PC and LPC by TLC

The assessment of the extent of reaction demanded a clean isolation of the reactant from the reaction mixture. This was achievable by (a) elution of the mixture from the microcolumn with methanol, (b) application of the eluate to

TABLE II

Extent of Reaction

Reaction time, min	A_2 per column, μl[a]	Number of experiments	Residual PC, %[b]
0[c]	---	6	92.8 ± 6.8
1	2	1	20.2
1	4	1	14.9
5	2	5	11.0 ± 1.8
5	4	5	12.6 ± 1.7
30	2	5	8.9 ± 1.1
30	4	4	7.9 ± 0.7
60	2	5	4.5 ± 1.3
60	4	5	3.3 ± 1.4
60	6	5	4.4 ± 1.0

[a]Volume of working solution of enzyme.
[b]See text for explanation. Multiple runs are reported as the mean ± standard deviation.
[c]Unreacted PC was applied to the plate and then analyzed in the presence of the adsorbent.

a TLC plate, (c) predevelopment of the plate with acetone to separate glycerol from the phospholipids, and (d) development in the acidic solvent system to separate LPC from PC.

Acyl Group Analysis

The selectivity of the new procedure was tested in two ways: (a) the solution that contained equal weights of $(14:0)_2$PC and $(18:2)_2$ PC was subjected to the reaction conditions in order to test the selectivity for unsaturated vs. saturated acyl groups; (b) the solution of α-16:0-β-14:0 PC was used to test the regioselectivity of the process for the β-acyl group.

The mixture of $(14:0)_2$PC and $(18:2)_2$PC was subjected to seven replicate 30 min reactions. GLC analysis of the methyl esters of the resulting fatty acids showed the 14:0 content to be 48.7 ± 1.6% by peak area, compared with the expected value of 48.8%, using the correction factors of Ackman (15). This indicated that the procedure was not selective for unsaturated vs. saturated acyl groups.

In order to test regioselectivity, several series of reactions were performed on aliquots of the α-16:0-β-14:0 PC in methylene chloride (25 μg of PC in 10 μl was applied to each column). GLC should have detected no palmitic acid if the model PC had palmitoyl groups only in the α-position and if no acyl group migration had occurred. Actual data are given in Table I. Regardless of the amount of enzyme present or the time of reaction, ca. 90 mole % myristic acid (14:0) was detected (molar mean ± standard deviation for the five sets in Table I = 89.7 ± 0.9) vs. 10% of the unexpected palmitic acid (16:0). Since specificity for the 2-acyl group did not diminish with longer reaction times and since it is shown in the accompanying publication (11) that this PC contained a 50:50 molar ratio of 14:0 to 16:0, the most likely cause for the appearance of 16:0 by enzymic hydrolysis was acyl group scrambling. Since the extent of scrambling did not increase with increasing reaction times, such scrambling probably occurred during the synthesis of the PC from α-16:0 LPC. In fact, some scrambling has been reported to have occurred in every mixed acid PC synthesis for which such analysis has been attempted (16,17). Furthermore, in an ongoing project in this laboratory, α-16:0-β-14:0 PC that was synthesized by a novel acylation of pure α-16:0 LPC showed 7% scrambling by application of the same microcolumn procedure that consistently showed 10% scrambling in the present report (18).

Extent of Reaction

Data on the rate of consumption of PC are given in Table II and were obtained as follows: for each experiment, three reactions of α-16:0-β-14:0 PC (25 μg in 10 μl CH_2Cl_2) were allowed to proceed concurrently and in an identical manner for a specific duration. The combined eluates were applied to a TLC plate and the residual PC was isolated by development of the plate. The combination of three reaction mixtures was necessary for accurate analysis of the traces of PC that remained after the first few minutes of reaction. The PC spot was analyzed for phosphorus content and compared with the phosphorus content of the initial and unreacted PC solution (i.e., of 3 x 10 μl) In order to assess the precision or repeatability of the procedure, each experiment generally was repeated several times, and the results were reported as mean values ± standard deviation.

Varying the amount of enzyme produced virtually no change in reaction rate. The reac-

tion approximated first order kinetics, independent of enzyme concentration, with a rate constant about 1.4 sec^{-1} (derived from $\ln[PC]-\ln[PC_o] = -kt$). The first order behavior is typical of many enzyme reactions involving a single substrate at low concentration (19). The reaction was rapid. Whereas over 95% of the PC was consumed within 1 hr, almost 90% reacted within the first 5 min.

For routine enzymic analyses, then, these reactions on microcolumns are a convenient and sensitive alternative to the methodology in use today. When the new procedure is invoked along with the total acyl group analysis by transmethylation on microcolumns (11), detailed structural assignment of the total acyl content of the lipid may be accomplished on microgram amounts of starting material.

REFERENCES

1. Desnuelle, P., in "The Enzymes," Chapter 19, Vol. VII, Edited by P.D. Boyer, Academic Press, New York, 1972, p. 575ff.
2. Hanrahan, D.J., in "The Enzymes," Chapter 4, Vol. V, Edited by P.D. Boyer, Academic Press, New York, 1971, p. 71ff.
3. Van Golde, L.M.G., and L.L.M. van Deenen, Biochim. Biophys. Acta 125:496 (1966).
4. Nutter, L.J., and O.S. Privett, Lipids 1:258 (1966).
5. Kates, M., in "Laboratory Techniques in Biochemistry and Molecular Biology, Vol. III, Part II, Edited by T.S. Work and E. Work, North Holland Publishing Co., Amsterdam, 1972, p. 568.
6. Christie, W.W., in "Lipid Analysis," Pergamon Press, Oxford, 1973, p. 273.
7. Horiuchi, Y., and S. Imamura, U.S. Patent 3,909,360, Sept. 30, 1975; Chem. Abstr. 81:101302c,165900d, 82:53480f,120941x.
8. Rakhimov, M.N., Sh. R. Mad'yarov, and A. Kh. Abdumalikov, Biokhimiya 41:569 (1976); Biochemistry (USSR) 41:470 (1976).
9. Blain, J.A., J.D.E. Patterson, C.E. Shaw, and M. Waheed Akhtar, Lipids 11:553 (1976).
10. Gubensek, F., Bull. Inst. Pasteur, Paris, 74:47 (1976).
11. Marmer, W.N. Lipids 13:835(1978).
12. Vaskovsky, V.E., E.Y. Kostetsky, and I.M. Vasendin, J. Chromatogr. 114:129 (1975).
13. Fales, H.M., T.M. Jaouni, and J.F. Babashak, Anal. Chem. 45:2302 (1973).
14. Schwartz, D.P., and R.S. Bright, Anal. Biochem. 61:271 (1974).
15. Ackman, R.G., in "Progress in the Chemistry of Fats and Other Lipids," Vol. 12, Edited by R.T. Holman, Pergamon Press, Oxford, 1972, p. 254ff.
16. Cubero Robles, E., and D. van den Berg, Biochim. Biophys. Acta 187:520 (1969).
17. Gupta, C.M., R. Radhakrishnan, and H.G. Khorana, Proc. Nat. Acad. Sci. USA 74:4315 (1977).
18. Marmer, W.N., J. Am. Oil Chem. Soc. 54:152A (1978) (abstract).
19. Cantarow, A., and B. Schepartz, in "Biochemistry," 4th ed., W.B. Saunders Co., Philadelphia, 1967, p. 217.